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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/435, C12N 5/06, C12P 21/00, A61K 38/02

A1

(11) International Publication Number: WO 95/19990

(43) International Publication Date: 27 July 1995 (27.07.95)

(21) International Application Number: PCT/US95/00929

(22) International Filing Date: 25 January 1995 (25.01.95)

(30) Priority Data:
08/186,806 25 January 1994 (25.01.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/186,806 (CIP)
Filed on 25 January 1994 (25.01.94)

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(81) Designated States: AU, CA, CN, FI, JP, KR, NO, NZ, RU, US. European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
With amended claims.

(54) Title: A NOVEL PROTEIN FROM *AEDES ALBOPICTUS* CELLS AND METHODS FOR ITS USE

(57) Abstract

The present invention provides a novel isolated and purified protein that is produced by *Aedes albopictus* L4.4 cells, having a molecular weight of 55 kDa of SDS-PAGE, is associated with lysosomal membranes and induces an anti-viral state. This anti-viral state is characterized by a total block of virus RNA synthesis with no effect on cell macromolecular synthesis. Also provided are various methods of using this novel protein or the gene encoding this protein.

A NOVEL PROTEIN FROM *Aedes Albopictus* CELLS AND METHODS FOR ITS USE

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of virology and protein chemistry. More specifically, the present invention relates to a novel 55 kDa membrane protein associated with lysosomes.

Description of the Related Art

Alphaviruses are membrane-containing plus polarity RNA viruses which are sustained in nature by a life cycle which includes vertebrates. Sindbis virus is the prototype of the alphaviruses. Alphavirus infection of tissue cultured mosquito cells results in an acute infection (accompanied by the production of a high concentration of virus) which is followed by a persistent phase of infection in which virus production occurs at a much slower rate. These persistently infected cells may be cultured indefinitely.

Alphaviruses are propagated in nature in both vertebrate and invertebrate hosts. Cultured cells of vertebrate and invertebrate origin are susceptible to infection by Sindbis virus, the prototype of the alphaviruses. Although tissue cultured cells differ in their ability to produce alphaviruses, most cell lines replicate alphavirus genomes and produce viral RNA.

Comparative studies of Sindbis virus replication in cultured vertebrate and invertebrate cells have revealed differences in the morphological and biochemical aspects of virus growth, as well as in the response of the host cells to virus infection.

The most striking difference in comparative studies on the growth of alphaviruses in cultured vertebrate and invertebrate cells is the cellular response to virus

infection. When cultured vertebrate cells are infected with an alphavirus, synthesis of host protein and RNA is rapidly terminated. High yields of progeny virions are accompanied by gross cytopathic effects, cell death, and lysis 10 to 20 hours post-infection. In contrast, after an initial acute phase of infection (during which yields of virus equivalent to those produced by vertebrate cells are realized), cultured mosquito cells survive infection by alphaviruses to produce persistent infections. Persistently infected mosquito cells produce virus at a much slower rate and may be cultured indefinitely.

Cultures of the U4.4 subclone of *A. albopictus* (mosquito) cells persistently infected with Sindbis virus produce a low molecular weight protein which, when applied to uninfected mosquito cells, results in the induction of an antiviral state. A similar activity has been identified in Semliki Forest virus-infected mosquito cells.

Using crude preparations of the Sindbis antiviral agent, the antiviral state was completely established after treatment of the cell for 48 hours and resulted in the inhibition of both 49S and 26S viral RNA synthesis when cells are subsequently infected with Sindbis. This Sindbis antiviral agent appears to be responsible for the low levels of virus production seen in persistently infected mosquito cell cultures. This antiviral protein (AVP) has been purified to homogeneity and is a very hydrophobic peptide of 3200 Da. Treatment of uninfected mosquito cells with this pure protein resulted in a temporary arrest of cell growth followed by return to normal growth patterns. Subsequently, the cells become refractory to infection with Sindbis virus and constitutively produce the antiviral peptide in the absence of virus or viral RNA. These unique virus-resistant mosquito cells (L4.4) have been passaged for 1.5 years without losing the virus-resistant phenotype.

The prior art is deficient in the lack of effective means of inhibiting the spread of certain viruses, including

alphaviruses. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a composition of matter comprising an isolated and purified protein that is secreted by *Aedes albopictus* L4.4 cells, having a molecular weight of 55 kDa of sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), is associated with lysosomal membranes and is produces an anti-viral state.

In another embodiment of the present invention, there is provided a pharmaceutical composition, comprising a composition of matter comprising an isolated and purified protein secreted by *Aedes albopictus* L4.4 cells, having a molecular weight of 55 kDa of sodium dodecyl sulfate-polyacrylamide electrophoresis, and a pharmaceutically acceptable carrier.

In yet another embodiment of the present invention, there is provided a method of preparing the protein of claim 1, comprising the steps of: growing *Aedes albopictus* (L4.4) cells in a media; harvesting the cells; and isolating and purifying a protein secreted by *Aedes albopictus* L4.4 cells, having a molecular weight of 55 kDa of sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) associated with lysosomal membranes from the media.

In still yet another embodiment of the present invention, there is provided a method of inhibiting the transmission of alphaviruses comprising the step of administering to a host infected with an alphavirus a pharmacologically effective dose of the composition of claim 2.

In another embodiment of the present invention, there is provided a method of inhibiting viral RNA synthesis comprising the step of administering to a host infected with a virus a pharmacologically effective dose of the composition of claim 2.

In yet another embodiment of the present invention, there is provided a transgenic mosquito formed by transfecting a mosquito with the gene coding for the protein of claim 1.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the comparison of [^{35}S] methionine/cysteine labeled total cellular proteins from U4.4 and L4.4 cells. Equal numbers of cells were labeled with ^{35}S methionine/cysteine (20 uCi/ml) for 48 hours, harvested and processed. Figure 1A shows the U4.4 cell light membrane fraction. Figure 1B shows the L4.4 cell light membrane fraction. Figure 1C shows the U4.4 cytosol. Figure 1D shows the L4.4 cytosol. Figure 1E shows the U4.4 cell P15 fraction and Figure 1F shows the L4.4 cell P15 fraction. Equal counts were loaded on each gel and electrophoresis was carried out in two dimensions; the first dimension was run from right to left and the second dimension was run from top to bottom. Arrow heads show the positions of P55.

Figure 2 shows the time course of induction of P55 by antiviral protein. Figure 2A shows the P15 fraction of nontreated U4.4 cells. Figure 2B shows the P15 fraction of U4.4 cells after 24 hour treatment with AVP. Figure 2C shows the P15 fraction of U4.4 cells after 48 hour treatment with

AVP. Figure 2D shows the P15 fraction from L4.4 cells as positive control. Cells were treated with AVP in [^{35}S] methionine/cysteine-containing medium (20 uCi/ml) for 24 or 48 hours prior to processing. Equal counts were loaded on each gel. Arrow heads show the positions of P55.

Figure 3 shows the fractionation of P15 fraction of *Aedes albopictus* cells on continuous density gradients. Equal amounts of total cellular proteins (produced as in Figure 1) were analyzed. The resuspended P15 fractions from U4.4 and L4.4 cells were centrifuged on Accudenz 20-40% gradients for 1 hour and 50 minutes at 52,000 x g. Gradients were fractionated and the fractions were assayed for lysosomal (filled squares), mitochondrial (+), peroxisomal (*) and plasma membrane (filled triangles) marker enzyme activities.

Figure 4 shows the cellular location of P55. Fractions from the gradients shown in Figure 4 corresponding to mitochondria and lysosomes were concentrated and analyzed by two dimensional gel electrophoresis. Figure 4A shows the U4.4 mitochondrial fraction. Figure 4B shows the L4.4 mitochondrial fraction. Figure 4C shows the U4.4 lysosomal fraction; and Figure 4D shows the L4.4 lysosomal fraction. Equal cpm were loaded on each gel. Arrows show the positions of P55.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, a 55 kDa lysosome-associated protein (P55) is described that is synthesized in L4.4 cells and is absent in U4.4 cells (the parent of the L4.4 cell line). There is a temporal relationship between establishment of the virus-resistant state and P55 synthesis.

The present invention is directed to a composition of matter comprising an isolated and purified protein that is secreted by *Aedes albopictus* L4.4 cells, having a molecular weight of 55 kDa of SDS-PAGE, is associated with lysosomal membranes and produces an anti-viral state.

It is specifically contemplated that pharmaceutical compositions may be prepared using the novel protein of the

present invention. In such a case, the pharmaceutical composition comprises the novel protein of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the novel protein of the present invention.

The level of ordinary skill of the average scientist in the area of molecular virology has increased substantially in recent years. A person having ordinary skill in this art would readily be able to sequence, without undue experimentation, the P55 protein. With the knowledge of the P55 protein, a person of ordinary skill would readily clone the gene encoding the P55 protein. This gene encoding the P55 protein would then be inserted into an appropriate expression vector and mosquitos would then be transfected with this vector. These transgenic mosquitos would then be incapable of transmitting the alpha viruses.

The present invention is also directed to a method of preparing the protein of the present invention, comprising the steps of: growing *Aedes albopictus* (L4.4) cells in a media; harvesting the cells; and isolating and purifying the protein of the present invention from the membranes of the L4.4 cells.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Preparation of L4.4 and U4.4 cells

Two *A. albopictus* (mosquito) cell lines were used. U4.4 cells were cloned and have been passaged in culture for 13 years. The L4.4 cell line was established and has been passaged in culture for 1.5 years. The U4.4 clone of *Aedes albopictus* cells was prepared from a culture of mixed larval cells provided by the Yale Arbovirus Research Unit. The cells were cloned by limiting dilution in micro titre plates such

that each well contained only one cell. The clone designated U4.4 was selected as the one clone showing best growth properties.

EXAMPLE 2

Purification of the Antiviral Protein

The L4.4 cells were produced by treating U4.4 cells with purified antiviral protein. The antiviral protein was purified from the medium of Sindbis virus-infected U4.4 cells. Two hundred milliliters of this medium was diluted with an equal volume of 0.01 M phosphate- buffered saline (PBS) at 4° for 72 hours using dialysis tubing with a 12 kDa molecular weight limit. The dialysate was tested for virus contamination by plaque assay and for antiviral activity by the assay described by Riedel and Brown, J. Virology, 29, 51-60 (1979). The dialysate was concentrated by freeze drying. The antiviral protein was purified serially by high performance liquid chromatography. The crude sample of AVP was passed through a Waters column (7.8 x 300 mm protein pak 60 [6,8-dihydroxy-4-oxaheptylsilyl-bonded amorphous silica]). The mobile phase was 16% 100 mM Tris-HCl, 4% 100 mM Tris base, 15% 1 M NaCl, 65% H_2O; conditions were 22°, 1.0 ml/minute flow rate at 1000 PSI and 0.30 absorption unit full scale (AUFS). The peak activity was further purified by ion exchange chromatography using a 10 x 100-mm protein pak (DEAE 8 hr AP1, Waters) column. The mobile phase was 0.1 M PBS (eluent A) and 1 M NaCl in 0.1 M PBS (eluent B) with a gradient of 0-100% B in 60 minutes, temperature of 22 degrees, 1.0 ml/minute flow rate at 1200 PSI, 0.1 AUFS. The peak containing the antiviral activity was finally purified by reverse phase HPLC on a 2.1 x 250 mm C18 (Vydac) column. The mobile phase was 0.1% TFA in water (eluent A) and acetonitrile (eluent B) with a gradient of 0-60% B in 40 minutes, temperature of 22 degrees, 0.25 ml/minute flow rate at 1200 PSI, 0.05 AUFS. The peak containing the biological activity represented an average yield of 15 micrograms of peptide. This fraction produced a

single band when run on a 16.5% tricine gel as described by Schagger and Jagow, Anal. Biochem., 166:368-379, 1987. This purified antiviral protein was used to treat U4.4 cells to produce L4.4 cells.

EXAMPLE 3

Culturing of the U4.4 and L4.4 cells

Both cell lines were cultured in the medium of Mitsuhashi and Maramorosch, as follows (in grams per 5 liters): (1) $CaCl_2$ x $2H_2O$, 1.0; (2) KCl, 1.0; (3) $MgCl_2$ x $6H_2O$, 0.5; (4) NaCl, 35.0; (5) $NaHCO_3$ (mixed individually in water and then added), 0.6; (6) $NaH2PO_4$ x H_2O, 1.0; (7) yeastolate, 25.0; (8) alpha-D-glucose, 20.0; (9) lactalbumin hydrolysate, 32.5; (10) phenol red (optional), 0.05. All ingredients were combined and stirred overnight at 4 degrees. After filter sterilization, the medium was stored at 4 degrees. The pH of the medium was adjusted in each bottle to 7.2 with sterile 7.5% sodium bicarbonate immediately before use. Then 20% FCS was added. The cells were cultured by passage in the media of Mitsuhashi and Masoamorosch at dilution of 1:10. The growth was at 28°C.

EXAMPLE 4

Treatment of mosquito cells with AVP

Treatment of mosquito cells with AVP was as follows: AVP was diluted into 9 ml of the fresh mosquito cell culture medium. This medium was then used to treat uninfected U4.4 cell monolayers at 28°C for 48 hours. The cells were then challenged with a multiplicity of infection of 100 PFU Sindbis virus. After one hour incubation, the cells were washed three times with medium to remove unabsorbed virus. The infected cells were incubated for 48 hours in fresh medium. The virus produced by these cells was titrated by plaque assay on BHK cell monolayers as described by Renz and Brown, 1978.

In studies examining induction of P55, cellular protein radiolabeling times were equalized while varying AVP tr atment times either by radiolabeling for 24 hours before

AVP was added to cell culture medium and continuing incubation for 24 hours (24 hour treatment). Alternatively, the radiolabel and AVP was added to cells at the same time, continuing incubation for 48 hours.

EXAMPLE 5

Fractionation of mosquito cell homogenates

Cultured mosquito cells were harvested, washed with ice-cold 10 mM PBS and incubated in ice-cold swelling buffer (10 mM HEPES, 15 mM KCl, pH 7.2) for 5 minutes. The swollen cells were centrifuged and homogenized in ice-cold breaking buffer (50 mM HEPES, 90 mM KOAc, 5 mM $MgCl_2$ and 10 mM PMSF, pH 7.2) with 32 strokes in a type A dounce homogenizer. Cell nuclei were separated by centrifugation for 10 minutes at 1000 x g. The postnuclear supernatant was centrifuged at 15,000 x g for 10 minutes and the pellet (P15 fraction) was resuspended in 1 ml of gradient solution (5 mM Tris-HCl, pH 7.6, 1 mM EDTA). The supernatant recovered from the P15 fraction was centrifuged for 2 hours at 100,000 x g. The pellet from this centrifugation was designated the light membrane fraction and the supernatant was designated the soluble portion of the cytoplasm. The P15 fraction was further fractionated for 1 hour, 50 minutes on a 20-40% (w/v) Accudenz (Nycodenz) gradient at 52,000 x g.

EXAMPLE 6

Enzyme assays

The following enzyme markers were used: beta-galactosidase (lysosomes), succinate p-iodonitrotetrazoliumviolet (INT) reductase (mitochondria), catalase (peroxisomes), and 5'-nucleotidase (plasma membranes). All the assays were carried out in 1.5 ml tubes. 0.1 ml samples containing the same protein concentration were used for each experiment.

Briefly, for beta-galactosidase the assay mixture contained 0.4 ml of 6 mM p-nitrophenyl beta-D-galactopyranoside, 0.5% Triton X-100, and 0.05 M citrate phosphate buffer, pH 4.3. After incubation at 37°C for 30

minutes, the reaction was terminated by the addition of 1 ml 0.25 M glycine-NaOH, pH 10. Sedimentable material was removed at 15,000 rpm for 1 minute and the absorbance of the supernatant was measured at 400 nm.

Succinate INT reductase was measured by incubation at 37°C for 10 minutes in 0.3 ml 0.01 M succinate in 0.05 M phosphate buffer, pH 7.5. After a second 10 minute incubation following addition of 0.1 ml INT (2.5mg/ml in the same buffer), the reaction was stopped by adding 1 ml ethyl acetate:ethanol:TCA (5:5:1) and the absorbance was measured at 490 nm.

Catalase was assayed by incubating the sample in 0.5 ml 6 mM H_2O_2 in 0.01 M phosphate buffer, pH 7.0, for 5 minutes at 0°C. Unreacted substrate was reacted with 0.01 N $KMnO_4$ (0.7 ml) after stopping the reaction with 0.1 ml 3 M H_2SO_4; the absorbance was measured at 480nm.

5'-Nucleotidase was measured in a total volume of 0.5 ml containing 2 mM [8-^{14}C]AMP, 0.4 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0. After incubation at 37°C for 30 minutes, the reaction was stopped by the addition of 0.3 ml 0.3 N $ZnSO_4$ and 0.3 ml 0.3 N $Ba(OH)_2$. The mixture was incubated at 0°C (with occasional agitation) for 15 minutes and the precipitate was removed by centrifugation at 0°C for 2 minutes; 0.2 ml of the supernatant was removed for counting.

EXAMPLE 7

Two dimensional gel electrophoresis

The membrane associated proteins and the concentrated soluble proteins from different cellular fractions were solubilized in sample buffer (9.5 M urea, 5% 2-mercaptoethanol, 2% nonidet P-40, 1.6% ampholine pH 5-8 [Preblended], and 0.4% ampholine pH 3-10). The first dimensional electrofocusing gel was made according to O'Farrell's method (O'Farrell, P. H. (1975), High resolution two-dimensional electrophoresis of proteins, J. Biol. Chem. 250, 4007-4021) and (O'Farrell, P. Z. et al., (1977). High-resolution two-dimensional electrophoresis of basic as well as

acidic proteins, Cell 12, 1133-1142 as described by Hames, B. D., and Rickwood, D. (ed) (1990), Isoelectric focusing and two-dimensional gel electrophoresis. In Gel electrophoresis of proteins, p.149-270. Oxford University Press, New York with minor modifications. Briefly, each 10.5 ml of the gel mixture contained: 5.5 grams of urea, 1.34 ml 28.38% acrylamide, 1.62% bisacrylamide, 2 ml 10% nonidet P-40, 0.4 ml 40% ampholine pH5-8, 0.1 ml 40% ampholine pH7-9, 0.1 ml 40% ampholine pH 3-10, 2 ml H_2O, 5 ul TEMED and 10.5 ul ammonium persulphate. The first dimensional electrofocusing gel was run in Hoefer tube gels for 4 hours at 500 volts after prefocusing for 1 hour at 250 volts. The electrolyte solutions were 10 mM H_3PO_4 (anolyte) and 20 mM NaOH (catholyte). Electrofocused gels were then soaked in equilibration buffer (2.5% SDS, 5 mM DTT, 125 mM Tris-HCl pH 6.8, 10% glycerol and 0.05% bromophenol blue) for 10 minutes. Equilibrated gels were placed on 10.8% SDS-PAGE slab gels and sealed with agarose (0.2% in 0.125 M Tris-HCl pH 6.8, brought to 2% SDS after cooling to 45°C). Second dimensional electrophoresis was carried out for 5 hours at a constant power of 5 watts. Fluorography was performed and gels were exposed to Kodak XAR-5 film.

EXAMPLE 8

The L4.4 Cell Line

The L4.4 cell line was incapable of replicating Sindbis virus RNA after infection with intact virions or transfection with genomic viral RNA. L4.4 cells were found to replicate a rhabdovirus (Vesicular Stomatitis Virus) as efficiently as the U4.4 (alphavirus-sensitive) cell line. Acquisition of the virus-resistant state was accompanied by the appearance of particular cellular proteins.

EXAMPLE 9

Radiolabeled cellular proteins from U4.4 and L4.4 mosquito cells

Cell homogenates from U4.4 and L4.4 mosquito cell clones were fractionated by multiple differential centrifugations. Three fractions of each cell line were

d signated: P15, light membranes, and cytosolic fractions and were compared to each other by two dimensional gel electrophoresis. A unique 55 kDa protein designated P55 was found in the P15 fraction of L4.4 cells (Figure 1F) which was not labeled in the comparable fraction of U4.4 cells (Figure 1E). P55 could not be detected in the light membrane or cytosolic fractions of either U4.4 or L4.4 cells (Figures 1A, 1B, 1C and 1D).

EXAMPLE 10

P55 is induced by the antiviral protein

The phenotypic differences between U4.4 cells and L4.4 cells are that L4.4 cells constitutively produce the antiviral protein and do not replicate Sindbis virus RNA. To illustrate the relationship between P55 synthesis and treatment with antiviral protein, [^{35}S] methionine/cysteine labeled U4.4 monolayers were treated with AVP for 24 or 48 hours and cell homogenates were fractionated. The P15 fraction produced from AVP-treated cells was subjected to two-dimensional gel electrophoresis. The P15 fraction from nontreated U4.4 cells served as A negative control and the P15 fraction from L4.4 cells served as a positive control.

Figure 2 demonstrates that after 48 hours of treatment of U4.4 cells with AVP, the amount of P55 reached about the same levels (Figure 2C) as that in the L4.4 control (Figure 2D). After 24 hours of treatment the amount of P55 detected was less than half the amount seen after 48 hours (Figure 2B). Thus, AVP treatment induces synthesis of P55. This result coincides with the fact that the virus-resistant phenotype is completely established at 48 hours after AVP treatment of susceptible cells. Thus, the increase in P55 concentration in the cells corresponds to the reduction in the ability of the AVP-treated cells to replicate viral RNA and to produce progeny virus. P55 cannot be induced by AVP treatment of baby hamster kidney (BHK) cells or C7/10 mosquito cells. This is consistent with the fact that the antiviral phenotype

cannot be established by treatment of BHK cells with AVP and that C7/10 cells neither produce nor respond to AVP.

EXAMPLE 11

Cellular localization of P55

P15 fractions from U4.4 and L4.4 cells were further analyzed by centrifugation through 20-40% continuous accudenz gradients. Two bands were observed, one at a density of 1.102 g/ml and the other at a density of 1.130 g/ml (Figure 3). Each fraction of the resulting gradient was assayed for four marker enzyme activities [Beta-Galactosidase (lysosomes), Succinate INT reductase (mitochondria), Catalase (peroxisomes) and 5'-Nucleotidase (plasma membrane)] as described in Example 6. The enzyme assays revealed that the two bands observed in the gradient represented lysosomes (fraction No. 3) and mitochondria (fraction No. 5). Catalase and 5'-Nucleotidase enzyme assays showed that these two major bands were not significantly contaminated by other cellular organelles. The gradient fractions containing lysosomes and mitochondria were collected and diluted with gradient buffer and centrifuged for 10 minutes at 15,000xg to pellet the lysosomes or mitochondria. The pelleted lysosomes and mitochondria were subjected to 2-D gel electrophoresis. The protein P55 was associated with the lysosomes of L4.4 cells (Figure 4D) but was absent in U4.4 cell lysosomes (Figure 4C) and the mitochondria of both U4.4 and L4.4 cells (Figure 4A and 4B).

The present invention shows that the virus-resistant L4.4 cells contain a 55 kDa lysosome-associated protein which is not detectable in virus-sensitive U4.4 cells. A direct relationship exists between treatment of virus-susceptible cells with AVP and the initiation of P55 production. P55 is present at concentrations equivalent to that found in L4.4 cells after a 48 hour AVP treatment of virus-susceptible U4.4 cells. P55 can be detected at lower concentrations after a 24 hour treatment with AVP. The relationship between P55 induction and acquisition of virus-resistance indicates that

P55 is directly involved in the inhibition of viral RNA synthesis.

The Sindbis viral RNA replication complex consists of viral plus and/or minus strand RNA, viral nonstructural proteins, nsP1, 2, 3 and 4 and some host proteins. Barton et al. (1991) isolated an alphavirus replication complex from the P15 fraction of infected baby hamster kidney cells. Such a complex has not yet been identified in insect cells. In the present invention, the P15 fraction from mosquito cells was found to contain mitochondria and lysosomes and P55 was found specifically associated with lysosomes.

In mammalian cells the alphavirus replication complex is associated with the cytoplasmic surface of lysosomal membranes. If this is also the case in mosquito cells, the presence of P55 may alter the properties of the lysosomal membrane such that the alphaviral RNA and nonstructural proteins cannot develop the membrane associations required for replication.

The present invention also comprises a method of inhibiting the transmission of alphaviruses. Given the benefit of the present disclosure, such a method would be readily developed by one having ordinary skill in this art. For example, the gene encoding the protein P55 would be identified and cloned. Subsequently, the protein P55 would be expressed in mosquitoes by a method described by McGrane, et al., Am. J. Trop. Med. Hyg., 39:502-510, 1988. Larvae which constitutively produce P55 protein are raised to adults. Adult mosquitoes are then resistant to infection by alphaviruses. Release of these insects into the wild will dilute the percentage of mosquitoes capable of carrying alphaviruses.

The present invention is also directed to a method of inhibiting viral, including alphaviral, RNA synthesis. The protein P55 is used to inhibit alphavirus RNA synthesis in cultured mammalian cells. Initially, insect cells which produce the protein P55 are fused with tissue-cultured

mammalian cells. The cell hybrid is infected with alphavirus (Sindbis) and production of virus RNA is determined. Thereafter, mammalian cells are transfected with cloned P55 DNA to produce a mammalian cell line which produces the P55 protein. The P55 protein is then available for use as an antiviral agent.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page Pages 3, 5, 6, 16, and 17, line ______ of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

American Type Culture Collection (ATCC)

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [5]	Accession Number [6]
09 August 1994	CRL 11699

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

G. Anton Smith
PCT International Division

(Authorized Officer)

17 FEB 1995

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

(January 1985)

Claims

1. A composition of matter comprising an isolated and purified protein that is secreted by *Aedes albopictus* L4.4 cells, having a molecular weight of 55 kDa of SDS-PAGE, is associated with lysosomal membranes and induces an anti-viral state.

2. A pharmaceutical composition, comprising the protein of claim 1 and a pharmaceutically acceptable carrier.

3. A method of preparing the protein of claim 1, comprising the steps of:

growing *Aedes albopictus* (L4.4) cells in a media at a temperature of about 28°C;

harvesting the cells; and

isolating and purifying the protein of claim 1 from said cells.

4. A method of inhibiting the transmission of alphaviruses comprising the step of administering to a host infected with an alphavirus a pharmacologically effective dose of the composition of claim 2.

5. A method of inhibiting viral RNA synthesis comprising the step of administering to a host infected with a virus a pharmacologically effective dose of the composition of claim 2.

6. A transgenic mosquito formed by transfecting a mosquito with the gene coding for the protein of claim 1.

AMENDED CLAIMS

[received by the International Bureau on 4 July 1995 (04.07.95); original claims 1 and 3 amended; remaining claims unchanged (1 page)]

1. An isolated and purified protein that is secreted by *Aedes albopictus* cells, having a molecular weight of 55 kDa as determined by SDS-PAGE, is associated with lysosomal membranes and induces a virus resistant state in *Aedes albopictus* cells.

2. A pharmaceutical composition, comprising the protein of claim 1 and a pharmaceutically acceptable carrier.

3. A method of preparing the protein of claim 1, comprising the steps of:

growing *Aedes albopictus* cells in a medium at a temperature of about 28°C;

harvesting the cells; and

isolating and purifying the protein of claim 1 from said cells.

4. A method of inhibiting the transmission of alphaviruses comprising the step of administering to a host infected with an alphavirus a pharmacologically effective dose of the composition of claim 2.

5. A method of inhibiting viral RNA synthesis comprising the step of administering to a host infected with a virus a pharmacologically effective dose of the composition of claim 2.

6. A transgenic mosquito formed by transfecting a mosquito with the gene coding for the protein of claim 1.

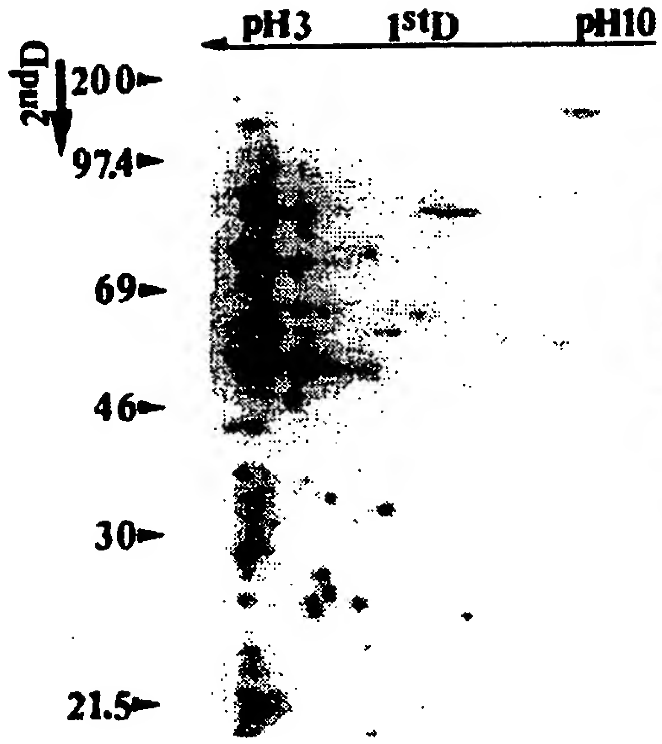

FIG. 1A

FIG. 1B

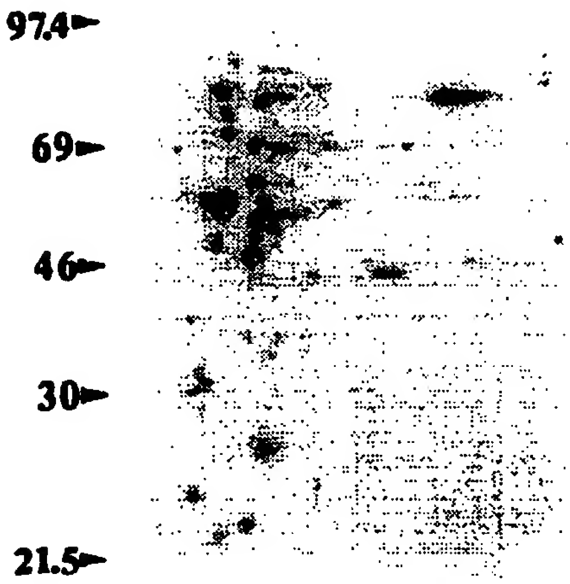

FIG. 1C

FIG. 1D

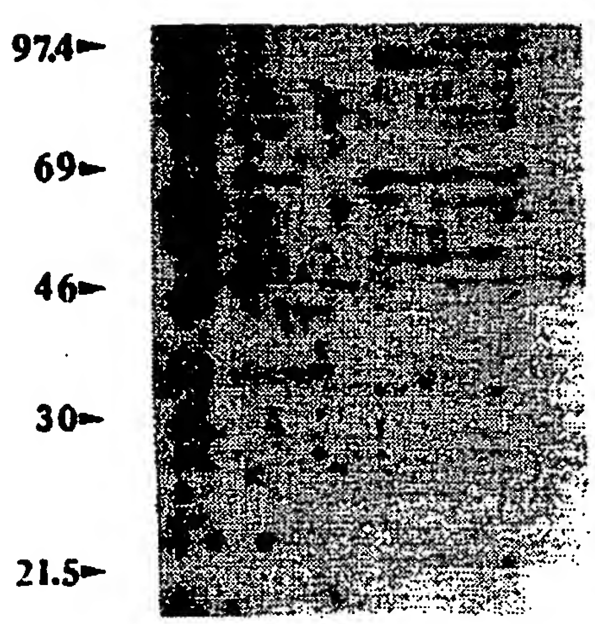

FIG. 1E

FIG. 1F

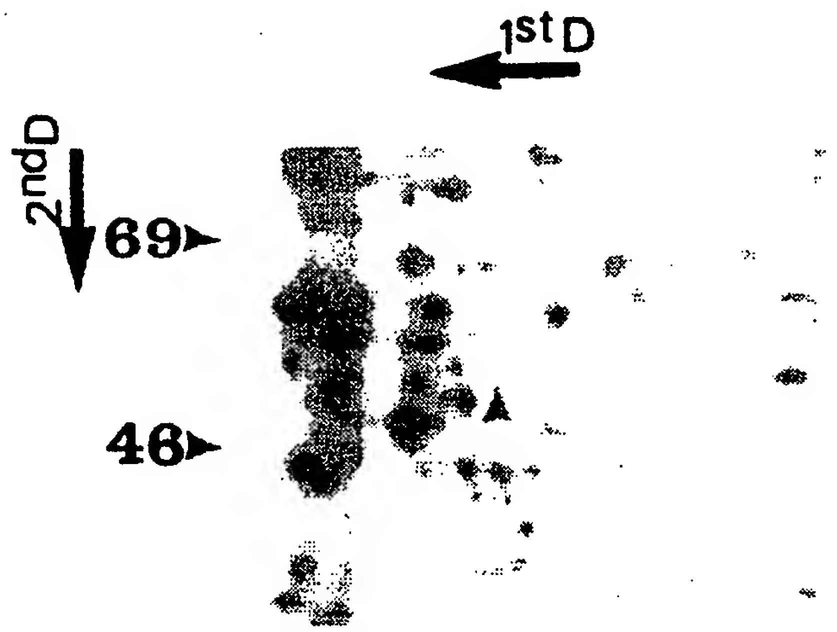

FIG. 2A

FIG. 2B

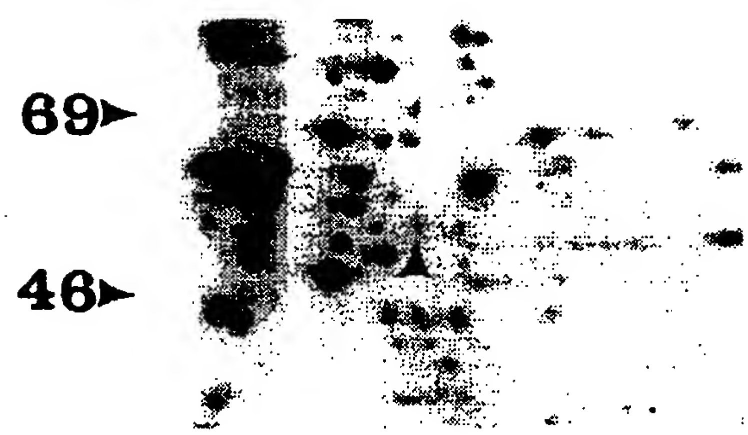

FIG. 2C

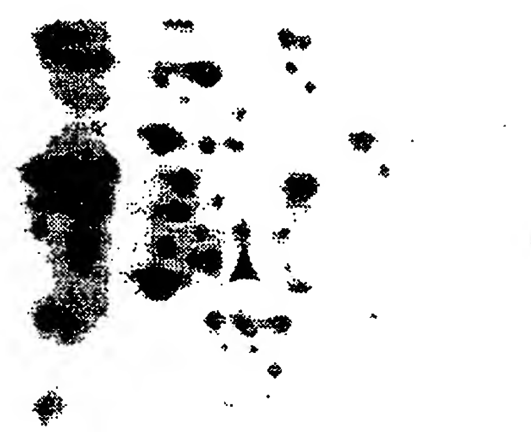

FIG. 2D

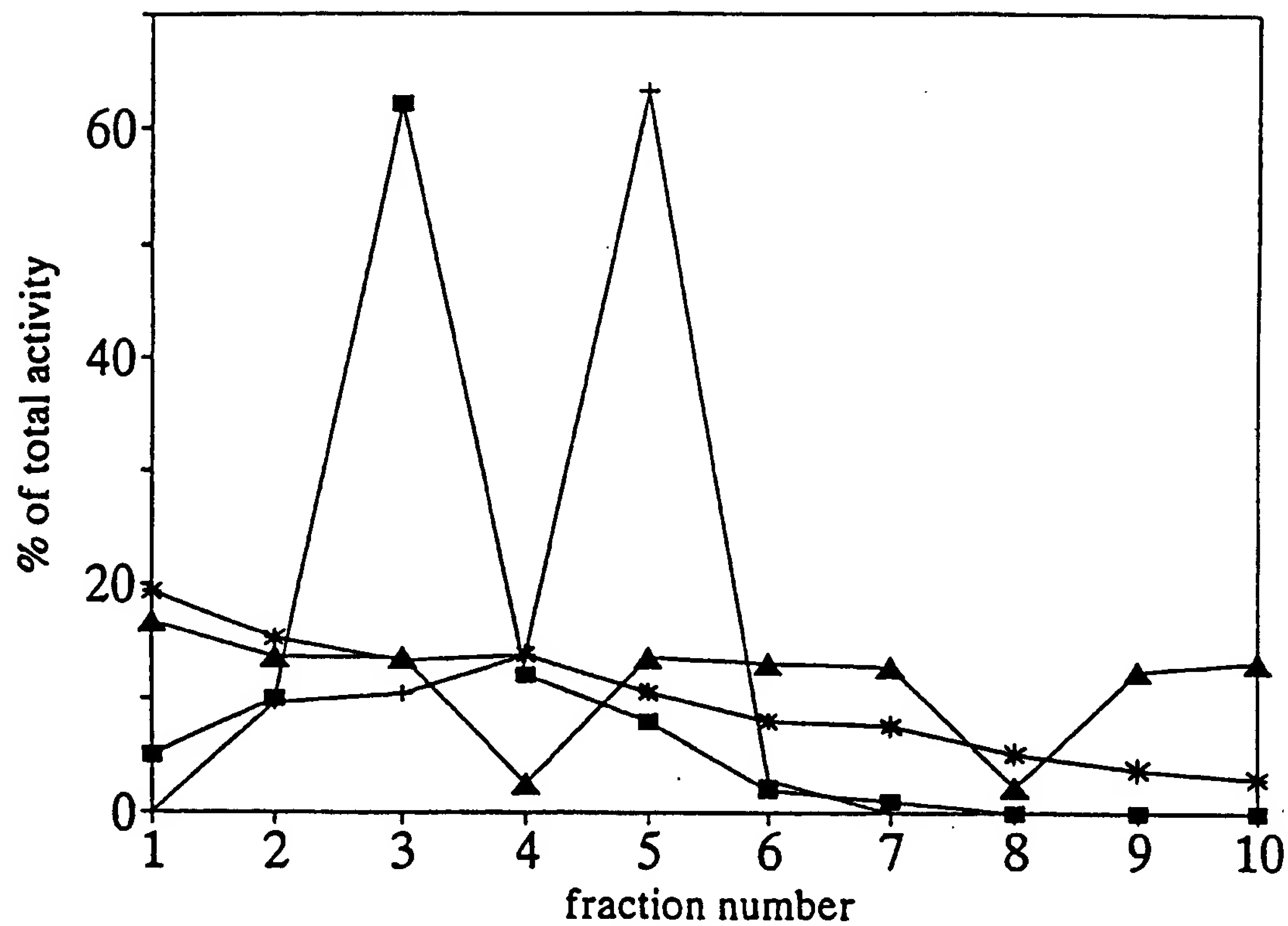

FIG.3

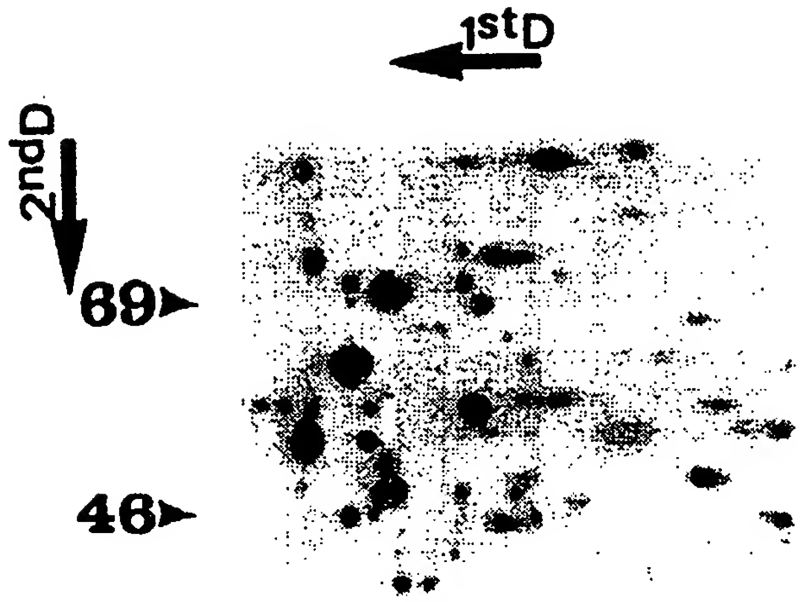

FIG. 4A

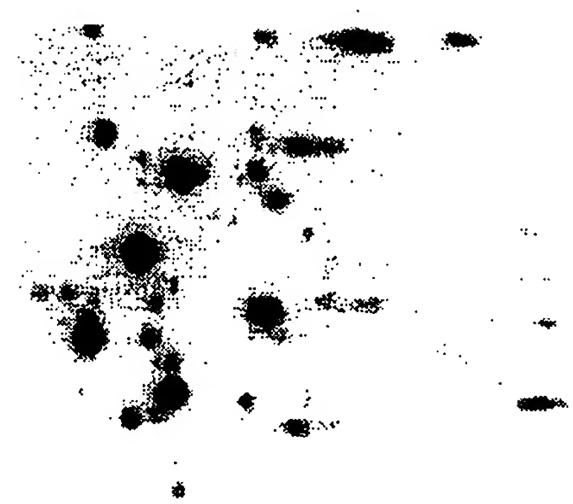

FIG. 4B

FIG. 4C

FIG. 4D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00929

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/435; C12N 5/06; C12P 21/00; A61K 38/02
US CL :435/70.3, 70.1, 172.3, 240.2; 800/2, dig 1, dig 2; 530/300; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/70.3, 70.1, 172.3, 240.2; 800/2, dig 1, dig 2; 530/300; 5 2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, WPIDS
Search terms: aedes albopictus, protein? alphavirus, virus?, mosquit?, virucidal, transgenic? treat?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 19, No. 21, Issued 1991, Morris et al., "FLP-mediated recombination in the vector mosquito Aedes aegypti", pages 5895-5900. See whole document.	6
Y	Parasitology Today, Volume 6, No. 2, issued 1990, Crampton et. al, "Transgenic Mosquitoes: A future Vector Control Strategy?", pages 31-36. See whole document.	6
X — Y	Virology, volume 194, issued 1993, Luo et al. "Purification and Characterization of a Sindbis Virus-Induced peptide which Stimulates Its own Production and Blocks Virus RNA Synthesis", pages 44-49. See whole document.	1-5 — 6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 MARCH 1995	08MAY1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	IRENE MARX
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal Of Virology, Volume 66, No. 7, issued July 1992, Miller et al., "Morphogenesis of Sindbis Virus in Three Subclones of Aedes Albopictus (Mosquito) Cells," Pages 4180-4190. See whole document.	1-6
Y	Proceedings National Academy of Sciences, Vol. 89, issued August 1992, Presnail et al., "Stable Genetic Transformation of a beneficial arthropod, Metaseiulus occidentalis (Acari: Phytoseiidae) by a microinjection technique," pages 7732-7736. See whole document.	6
Y	American Journal of Tropical Medicine and Hygiene, Vol. 35, No. 5, issued 1988, McGrane et al., "Microinjection of DNA into Aedes Triseriatus Ova and Detection of Integration", pages 502-510. See whole document.	6
Y	Medical and Veterinary Entomology, Vol. 3, issued 1989, Morris et al., "Genetic Transformation of the mosquito Aedes aegypti by micro-injection of DNA", pages 1-7. See whole document.	6

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5, drawn to an Aedes albopictus protein, a process of making the protein and a process of using the protein.
Group II, claim(s) 6, drawn to a transgenic mosquito formed by transfecting a mosquito with a defined gene.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The composition of matter of Group I is not an integral part of the transfection process required to make the product of group II. The process of Group I is not required for the manufacture of the product of Group II. In addition the products of groups I and group II, a protein and a mosquito are distinct and independent products lacking the same or corresponding technical feature.